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Unexplored Metabolomics Role in *Santalum album* L. Rhizosphere for Soil Fertility

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Abstract

Sandal (*Santalum album* L.) is an economically important tropical tree species, owing to extensive logging, changes in land-use patterns and poor natural regeneration, the natural sandal populations are rapidly dwindling. It is feared that such threats could easily undermine the genetic diversity of sandal populations and effective measures to prevent such loss are prerequisite. This study was measured the total phenolics contents were analyzed, the functional groups and chemical compounds using FT-IR and GC-MS. The phenolic acids in rhizosphere soil were extracted and detected by GC-MS, like 2, 4-Di-tert-butylphenol, 2-Butenedioic acid (Z), dibutyl ester, 1, 2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester, Diethyl Phthalate and Phthalic acid, butyl nonyl ester were found to have different content changes. Phthalic acid, butyl nonyl ester having antifungal, antiviral and antibacterial activities. Diethyl Phthalate having auto toxicity nature, which may inhibit the growth, as well as effects of the above phenolic acids and esters on the growth of sandal. This study presents a simple untargeted metabolomics workflow for extraction and an approach to estimate microbial metabolite available in rhizosphere soils. We have developed baseline data on the levels of metabolites distributed on *S. album* rhizosphere soil. This study was aimed to determine the metabolites that have the strongest relationship with sandalwood. Thus, GC-MS and FTIR has the potential to impact soil science by investigating the adaptable soil metabolomes in forestry fields, knowledge of which could be used to improve sandal production.

***Key words*:** Sandal tree, *Santalum album* L., Rhizosphere, FT-IR, GC-MS, Metabolomics, Soil fertility

Sandal (*Santalum album* L.) is an endemic East Indian sandalwood tree, which is sought for its fragrant heartwood and essential oil. Due to heavy commercial exploitation, the number of populations of sandal in their native habitat has significantly decreased [1], with its hemiparasitic nature initial for establish, sandalwood growth is strongly influenced by its surroundings, particularly the soil and host plant [2-3]. Sandalwood and the host plant would be physically, morphologically and physiologically connected via the created haustorium. Thus, the connection would permit the parasite plant to receive nutrients and water from the host plant [4]. In addition to complex microbial communities, soil is made up of both organic and inorganic chemical components, collectively known as the soil metabolome [5]. Microbes have a role in the biological complexity of soil and the cycling of nutrients. There is a lot of research being done on how crop/forest tree production interacts with the soil metabolome. Little is understood about how the soil metabolome as a whole affects plant disease resistance, despite ongoing attempts to characterize the soil microbiome using genetic techniques, which reveal information on the composition of the microbial community [6].

Metabolomics is the generic name assigned to a scientific field that addresses the characterization of low molecular weight organic metabolites released by living organisms in response to environmental stimuli. The difficulty of detecting a large number of metabolites and the requirement for developing metabolite databases specifically devoted to environmental challenges are both highlighted [7]. Metabolites are indispensable component of plant metabolism owing to their influence on plant biomass and architecture [8]. In secondary metabolites are low molecular weight organic compound involved in communication between microbial communities and host plants [9]. Some compounds that are introduced into the rhizosphere cannot be broken down by microbes and build up to a certain amount, posing a threat to plant growth [10]. In order to accomplish the impact of plant inhibition, particular auto toxic compounds are reversibly absorbed by soil.

Consequently, research into autotoxicity is crucial to understanding how continual cropping obstacles work [11]. Currently, the allelochemicals released into the rhizosphere soil by root secretion, leaching, decomposition and other means, mostly include esters, phenolic acids, and alkanes are intimately related to the study of ongoing planting barriers of sandal plants [12]. Hence, the present investigation is to focus the metabolomics role for soil fertility in sandal rhizosphere domain.

MATERIALS AND METHODS

Sample collection

A one-year-old sandal tree without host plant rhizosphere soil sample was collected from RBL Nursery Field

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at Bharathidasan University (10°40′29″N 78°44′39″E) in the Tiruchirappalli district of Tamil Nadu, India. The study was conducted in 2021–2022. The experimental site has a semi-arid environment with 387 mm of annual precipitation distributed as drier June through September, wetter January through February, hotter April through May, and cooler October through December. Three sandal saplings are planted at once in different ways. One sapling planted without host, second one with *Actionobacteria* treated seeds without any host and the third one with biofertilizer treated with *Actionobacteria.* According to morphology basis we chosen *Actionobacteria* treated one year sandal tree for sample processing. The research area's soil was a low-fertility, red soil with a pH of (5.4). The drip irrigation system offered on summertime protective irrigation for the plantation. The collected soil sample was kept under shadow dry for one week. After drying the soil sample was homogenized and sieved with the help of 2mm sieve mesh. The fine powdered soil sample was kept at -20 °C.

Soil extraction

Collected rhizosphere soil extraction were performed using LC-MS grade water (pH 7.4), 10 mM K_2SO_4 (pH 6.3), 10 mM NH_4HCO_3 (pH 8.5), isopropanol/methanol/ water (3:3:2 v/v/v) or 10-100% methanol. All extraction solutions were sterilized by filtration through 0.2 mm cellulose acetate filters. Field-moist soil (2 g, unfumigated or fumigated) was added to 50 ml polypropylene Falcon tubes and kept on ice. Ice cold extraction solution (8 ml) was added followed by a spike with the internal standard, ABMBA (8 mg). Sample were shaken on an orbital shaker at 200 rpm for 1 h at 4℃, and then centrifuged at 3220 rpm for 15 min. The supernatant was filtered through 0.45 mm syringe filters into 15 ml tubes and dried in a Savant Speed Vac SPD111V up to 8 hrs. Dried residues were resuspended in 200 ml methanol, vortexed and sonicated for 5 min followed by a final filtration through 0.22 mm centrifugal membranes and an aliquot (100 ml) dried for GC-MS derivatization.

ATR- FTIR spectrum analysis

For soil analysis, Fourier transform infrared (FTIR) and attenuated total reflectance spectroscopy (ATR) are frequently used [13]. For ATR- FTIR spectrophotometry analysis, 8 mg of dried soil sample was mixed with 1 ml of methanol. A handheld Jasco Fourier transform infrared spectrometer - 4600 was used to scan attenuated total reflectance (ATR) spectra (Jasco Corporation, Japan). A spectral resolution of 4 cm^{-1} was used to capture spectra in the range of 4000 to 550 cm^{-1}. For better contact, the soil sample was placed up against the diamond reflecting element. A blank reference was scanned first, and then the soil sample.

Conditions for chromatography and mass spectrometry
GC chromatographic conditions

The flow rate is 1.2 mL/min, and the tail blowing flow is 60 mL/min, total flow 1.2 mL/min, post air flow 1 mL/min. Injection port temperature 180 ◦C, injection volume 1 µL, no split injection. Column temperature program, -60 °C - 325 °C (350 °C).

GC-MS conditions

The constant current mode, the flow rate is 1.2 mL/min, the inlet temperature is 260 ℃, the injection volume is 1 µL, there is no split injection, the GC-MS interface temperature is 300 ℃, the ion source temperature is 230 ℃, the quadrupole temperature is 150 ℃, the ionization mode is EI, the electron energy is 70 eV, and the multiplier voltage is added 400 V after automatic tuning. Column temperature procedure, 60 °C - 325 °C (350 °C). This study was to investigate the growth of *S. album* tree and their rhizosphere soil for separation, and analyze the content change of phenolic acids and ester compounds, so as to judge whether phenolic acids and ester compounds had allelopathic effect on the growth of *Santalum album*.

GC-MS data acquisition

Metabolites were recorded for *S. album* rhizosphere soil sample using GCMS. Data acquisition was carried out by the GCMS SCAN_B.amx method. The peak area in a GC-MS chromatogram was automatically integrated and corrected through the NIST library. The resulting peak lists were submitted to the NIST library. The results were manually verified by analyzing Matching ratio and Uniqueness parameters and cross referenced with data available on the NIST library. The chemical shift assignment for each peak was recorded and compared to hits from the known databases for compound identification. Chemical identities were confirmed using GC-MS from NIST.

Statistical analysis

Statistical analyses were carried out using Origin Pro. Score plots were prepared with OriginPro 2019b (32-bit), using the average and standard deviations determined for sandal plant whose seeds were treated with *Actinobacteria*.The organization of samples in PCA scores plots is based on the similarities between their metabolic profiles.

RESULTS AND DISCUSSION

According to morphology aspects, *actinobacteria* treated seeds showed high quality results when compared with other two experimental observations. After comparison high morphology sandal tree rhizosphere soil was taken for metabolites identification. Complex metabolite mixtures were obtained by extraction of the soil sample from methanol as an evidenced by comparison of representative GC-MS and FT-IR. During GC-MS analysis the predominant numbers of peaks were observed in regions of the spectra through NIST17 library where phenols and ester compounds were identified. FT-IR analysis revealed the presence of amines, alkane, aldehyde, alcohol, and ether. Accordingly, regulatory programs worldwide are currently incorporating tests with endpoints that involve the effects of chemicals and the impact in specific metabolic pathways. Toxicological end-points can be general biological responses such as survival or weight loss. According to this several metabolites arise from the *S. album* field had some toxic nature and other metabolites had growth promotion in soils listed in (Table 1).

Table 1 Band assignments in FTIR spectra of *S. album* and its methanol extracts

Wave number cm^{-1}	Functional group	Compound class
3790.4	O-H stretching	Alcohol
3662.16	O-H stretching	Alcohol
3321.78	N-H stretching	Secondary amine
2944.77	C-H stretching	Alkane
2347.91	O=C=Stretching	Carbon dioxide
1737.55	C=O stretching	Aldehyde
1366.32	S=O stretching	Sulfonamide
1216.86	C-O stretching	Vinyl ether
1020.18	C-N stretching	Aliphatic amine

Techniques based on Gas chromatography mass spectrometry are certainly required to understand the

mechanisms involved in the alteration of metabolic pathways as response to autotoxicity. (Fig 1) illustrates the change in the fingerprint of organic compounds in a *S. album* soil with different sources. While some of the groups of compounds might be merely related to the sources of carbon added (550-4000 cm^{-1}) can be associated to changes in the metabolic fingerprint of the *S. album* soil system and therefore linked to microbiological activity in soil. Overall, the introduction of these results seeks to encourage further characterization of families of compounds in intact soil or function in relation with soil processes, an approach that can find immediate application in the assessment of biological responses to toxic compounds in *S. album* soil. The variability of biological responses has been one of the main obstacles for their implementation in standardized risk assessment. However, the examination of changes in biological processes by accurate analytical techniques had launched a new era in our understanding of the soil processes. The possibility of identifying the most sensitive metabolites of *S. album* may alter their function in growth and development.

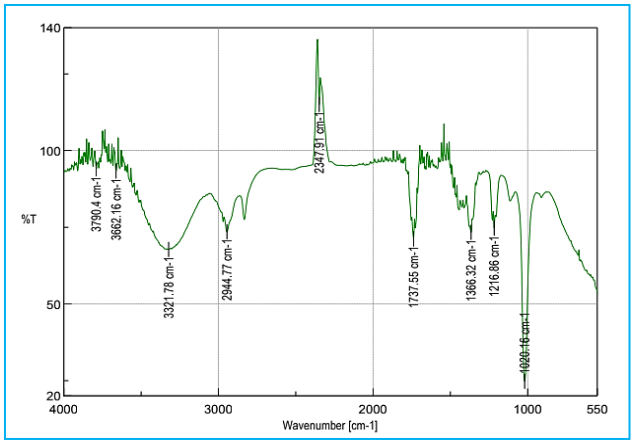

Fig 1 Absorption spectra obtained by Fourier transform infrared spectroscopy (FTIR) for *S. album* soil

Chemical constituents identified in the methanol extracts of *S. album* rhizosphere soil are presented in (Table 2). 2,4-di-tert-butylphenol (2,4-DTBP) (42.87%), 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (4.7%),2-Butenedioic acid (Z), dibutyl ester (58.63%), Diethyl Phthalate (79.76%) and Phthalic acid, butyl nonyl ester (6.8%) were the major constituents identified from the methanol extracts of *S. album* rhizosphere soil (Fig 2). Compared with literatures out of five metabolites, majorly 2, 4-di-*tert*-butylphenol in *S. album* may show the positive effect on growth and development and diethyl phthalate may shows toxicity in *S. album.* 2, 4-Di-tert-butylphenol showed the peak at 15.964. 2-Butenedioic acid (Z)-, dibutyl ester showed the peak at 16.580. Diethyl Phthalate showed the peak at 17.909. 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester showed the peak at 24.157. Phthalic acid, butyl nonyl ester showed the peak at 26.788.

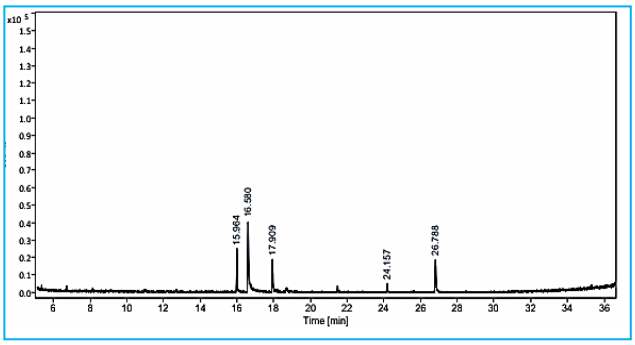

Fig 2 GC-MS analysis of *S. album* rhizosphere soil from methanol extracts

The five phenolic contents in *Santalum album* varied depend on their growth and the plant morphology. The phenolics compounds are all accumulated in the rhizosphere part of young sandal tree plant. In this study, we experimented with three sandal trees. First sandal sapling was planted without host, secondly sandal seeds were coated with *Actinobacteria* culture and planted without host and finally the third sapling was planted without host and treated with biofertilizers + *Actinobacteria*. The cultured soil nutrient condition might influence the phenolic content in one year old *Santalum album* tree sample, the soil moisture, and the growth time. According to reports, the ability to withstand environmental biotic and abiotic stimuli is greatly influenced by the accumulation of secondary metabolites. These secondary metabolites aid stressed plants in adjusting to various environmental factors, such as the antioxidant phenol and flavonoid accumulation [14]. However, in this study we found that *Actinobacteria* treated sandal tree shows high girth and height compared to the untreated sandal tree morphology aspects.

Table 2 Identified metabolites of methanol extracts of *S. album* rhizopshere soil

S. No.	Names of compounds	CAS	Molecular weight (g/mol)	Formulas
1.	2,4-Di-tert-butylphenol	96-76-4	206.32	$C_{14}H_{22}O$
2.	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	84-69-5	278.3435	$C_{16}H_{22}O_4$
3.	Diethyl phthalate	84-66-2	222.24	$C_{12}H_{14}O$
4.	2-Butenedioic acid (Z)-, dibutyl ester	105-76-0	228.2848	$C_{12}H_{20}O_4$
5.	Phthalic acid, butyl nonyl ester		348.5	$C_{21}H_{32}O_4$

However, for screenings which merely require the detection of differences between metabolomic methods such as FT-IR would be suitable, particularly if extremely high sample throughput is required [15]. The FTIR spectra derived from *Actinobacteria* treated sandal tree soil sample showed a difference in functional grouping. Cultured sandal tree having differences in the content level of metabolites. The functional groups obtained from the extracts of *Actinobacteria* treated sandal tree soil sample are S=O, C=O, C–O, C–N, C=C, –CH, –NH, and –OH, which are likely to be associated with antioxidant activity [16].

2-Butenedioic acid (Z), dibutyl ester, Diethyl Phthalate and Phthalic acid, butyl nonyl ester are previously reported in heartwood GC-MS analysis [17]. 2, 4-DTBP was found in 16 species of bacteria in 10 families, such as nitrogen-fixing cyanobacteria [18]; Gram-positive bacteria in hot spring, soils, and food [19-24] and gram-negative bacteria in soil and freshwater [25-30]. 2, 4-DTBP can be produced in some species of *Aspergillus* [31], *Penicillium* [32-34] and *Fusarium* [35], but experiments showed the phenol could inhibit the growth of these fungi. 2, 4-DTBP shows potential as a natural and environmentally friendly herbicide for weed management [36].

2, 4-DTBP has potent herbicidal properties that can alter the chloroplast ultrastructure, thereby reducing physiological activity of these weedy plants [37]. The findings imply that 2, 4-DTBP may potentially be developed as a soil-applied natural

herbicide for the control of *L. chinensis* and perhaps other weeds in an aerobic rice system [38-39]. Previous studies have confirmed that 2, 4-di-*tert*-butylphenol (2, 4-DTBP) is a major autotoxin of the root exudates that severely hampers the yield and quality of *Lanzhou lily* [40]. It was found that 2, 4-DTBP primarily accumulated in the transition tissues between the heartwood and sapwood as the major component in the ethyl acetate extracts [41]. The bioactive metabolites of nodule associated microbes (NAM) revealed the existence of several soluble metabolites which includes 2, 4-Di-tert-butylphenol, 1, 2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester and phthalic acid butyl hex-3-yl ester were identified in Shirley Evangilene *et al*. [42]. 2-Butenedioic acid (Z)-, dibutyl ester shows antibacterial activity in *Kochia indica* Wight [43].

There is a great need to understand the environmental impacts of organic pollutants on soil health. Phthalates are widely used in consumables and can be found extensively. Diethyl phthalate (DEP) has a significant effect on microbial communities and subsequently potential consequences for environmental processes. Chemicals in the environment and their effect on soil health have led to increasing interest in monitoring acute and chronic toxicity and mutagenicity in soil [44-46]. Diethyl phthalate is a man-made colorless liquid with a slight aromatic odor and a bitter, disagreeable taste. Diethyl phthalate is also used in cosmetics, insecticides, and aspirin. Diethyl phthalate is fairly mobile in soil, based on tests of the absorption of diethyl phthalate from double-distilled water onto composite soil (1.59% organic carbon); diethyl phthalate moved through the soil at half the rate of water [47].

CONCLUSION

In the present study, we investigated the role of 2, 4-DTBP alone on soil enzymes and microorganisms in the rhizosphere soil of *Santalum album*. In this study, 2, 4-DTBP might be resulted as growth compound for *Santalum album* rhizosphere soil, which may be one of the mechanisms that trigger growth of *Santalum album.* The present investigation found that GC-MS analysis of the methanol extract of rhizosphere soil sample from *Santalum album* revealed the presence of multiple bioactive constituents responsible for sandal growth and development. One metabolite shows toxic effect which may affect the sandal growth compared to other literatures. GC-MS analyses of rhizosphere soil extract depicting the significant role of bioactive compounds in plant growth promotion. Successful planting practices would be encouraged upon formulating ecofriendly soluble metabolites responsible for beneficial effects on plant growth under extreme environments and constructing inbuilt resistance against phytopathogens. These findings provide a basic understanding of the autotoxins and the bioactive metabolite compounds between *Santalum album* and the rhizosphere soil, which is significant for understanding the growth mechanism of *Santalum album*.

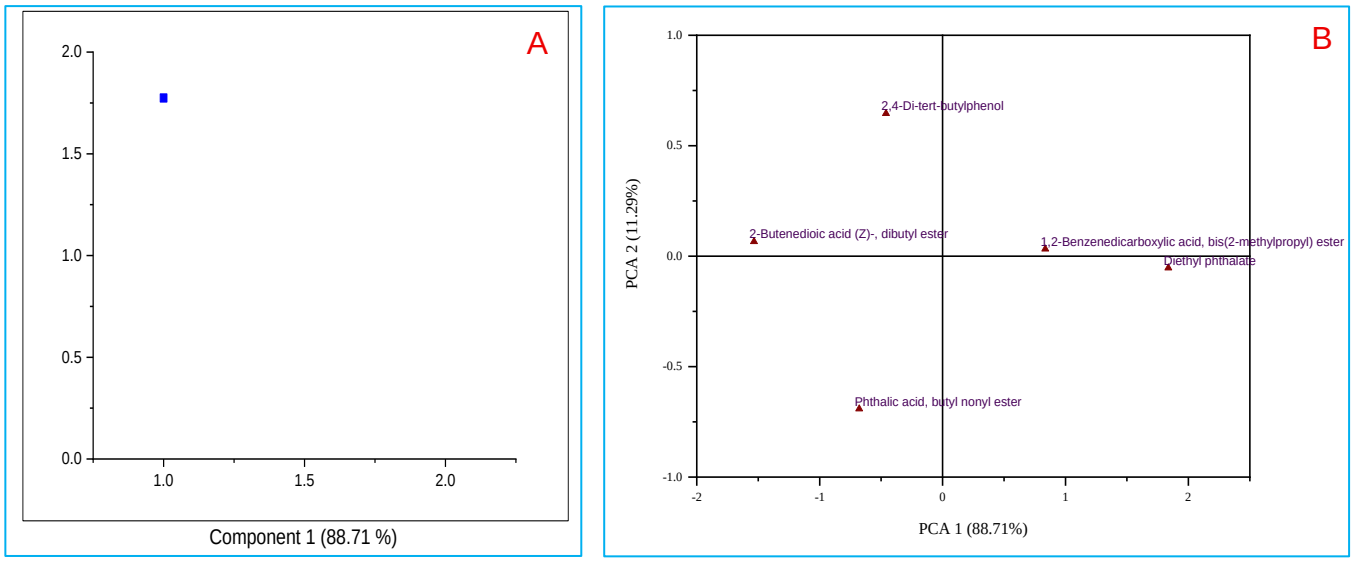

Fig 3 Multivariate analysis of extracted metabolites from *actinobacteria* treated seeds rhizosphere soil. Principle component analysis (PCA) score plot (A) demonstrating the *actinobacteria* treated seeds rhizosphere soil metabolites

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